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Neisseria gonorrhoeae Filamentous Phage NgoΦ6 Is Capable of Infecting a Variety of Gram-Negative Bacteria

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We constructed a phagemid consisting of the whole genome of the *Neisseria gonorrhoeae* bacteriophage NgoΦ6 cloned into a pBluescript plasmid derivative lacking the *f1* origin of replication (named pBS::Φ6). *Escherichia coli* cells harboring pBS::Φ6 were able to produce a biologically active phagemid, NgoΦ6fm, capable of infecting, integrating its DNA into the chromosome of, and producing progeny phagemids in, a variety of taxonomically distant Gram-negative bacteria, including *E. coli*, *Haemophilus influenzae*, *Neisseria sicca*, *Pseudomonas* sp., and *Paracoccus methylutens*. A derivative of pBS::Φ6 lacking the phage *orf7* gene, a positional homolog of filamentous phage proteins that mediate the interaction between the phage and the bacterial pilus, was capable of producing phagemid particles that were able to infect *E. coli*, *Haemophilus influenzae*, *N. sicca*, *Pseudomonas* sp., and *Paracoccus methylutens*, indicating that NgoΦ6 infects cells of these species using a mechanism that does not involve the Orf7 gene product and that NgoΦ6 initiates infection through a novel process in these species. We further demonstrate that the establishment of the lysogenic state does not require an active phage integrase. Since phagemid particles were capable of infecting diverse hosts, this indicates that NgoΦ6 is the first broad-host-range filamentous bacteriophage described.

Filamentous bacteriophages belong to three taxonomic families: *Inoviridae*, *Lipothrixviridae*, and *Rudiviridae* (1). The family *Inoviridae* is represented by two genera: *Inovirus* and *Plectivirus* (1). Bacteriophages of the genus *Inovirus* infect almost exclusively Gram-negative bacteria, and their capsid is in the form of a long (750-nm to 2,000-nm) filamentous structure (2). They can also be divided into two subgroups: (i) those able to integrate their genomic DNA into the bacterial genome, represented by a broad range of phages like CTXΦ (3), YpfΦ, or ΦRSM (4) infecting *Vibrio cholerae*, *Yersinia pestis*, and *Ralstonia solanaceum*; and (ii) those unable to integrate their genomic DNA into the chromosome of the host bacterium, represented by the bacteriophages fd, f2, and M13 (2). The *Neisseria gonorrhoeae* phages NgoΦ6 and NgoΦ7 (5, 6), along with *Neisseria meningitidis* phage Nmf (7), share homology with the first group.

The genomes of all *Inoviridae* phages are organized into three modules in which functionally related genes are grouped together (1–8): the replication genes (gII, gV, and gX); the structural genes (gIII, gVI, gVII, gVIII, and gIX); and the assembly and release genes (gI and gIV). Among the structural genes, gIII (or its equivalent) encodes the host recognition and adsorption protein pIII, and this protein is required for efficient infectivity. Bacterial pili specifically recognize and bind this minor coat protein at the initiation of infection. The key protein in the assembly and release module, pIV, produces an aqueous channel in the outer membrane through which phage particles exit from the host cell. Filamentous phages that can integrate their DNA into the bacterial host genome use the XerC and XerD recombinase/resolvase of their host for integration (9, 10) or use integrases that they encode (11), as was suggested for *N. gonorrhoeae* filamentous phages (5).

The frequency of horizontal transfer can be limited by the specificity of the phage receptor protein pIII. Changing the pIII protein can alter the host range of filamentous phage. This was demonstrated for coliphage fd, which can use $pIII^{CTX}$ to expand the host range to include *Vibrio cholerae* (12), and for *Yersinia pestis* phage YpfΦ, which is able to replicate not only in different strains of *Yersinia* but also in *Escherichia coli* (12). Horizontal transfer of filamentous phages across bacterial species could be very important, taking into consideration their role in the pathogenicity of bacteria (13–16).

The filamentous phages M13, f1, and fd have been used to form a variety of cloning vectors, designated phagemids, best exemplified by pBluescript plasmids (17). Phagemids were developed as a hybrid, containing DNA sequences from a filamentous phage and a plasmid, to produce a vector that can replicate as a plasmid but also be packaged into viral particles as single-stranded DNA (ssDNA). Phagemids contain an origin of replication (*ori*) for double-stranded replication as well as a filamentous phage DNA *ori* to enable ssDNA replication. Phagemids typically lack the genes required for packaging of the phage ssDNA into virions, and "helper" bacteriophage are needed to provide these functions. The M13 family of phagemids are derivatives of the single-stranded, male-specific filamentous DNA bacteriophage M13 (18–20). All of the phage functions, such as synthesis of viral (+) ssDNA, of the structural proteins, and of proteins required for phage particle assembly and release, are encoded by phage DNA. The ssDNA synthesis is initiated at the ori (+) sequence.

In this paper, we describe the construction and characterization of a phagemid derived from the *N. gonorrhoeae* filamentous phage NgoΦ6. We further demonstrate that isolated phagemid particles contain ssDNA that can infect, establish a state of lysogeny in, and replicate in several bacterial species other than their

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TABLE 1 Primers used in this study

Primer	Sequence (5′ → 3′)	Use
Phi6 F	ATGGATCCTACCGTTGAAGCCAATTTGGCACTCG	Cloning of Φ6 genome DNA
Phi6 R	ATGGATCCACTCTTTTCCCGTACCGCCAACCTC	
mpm11p	GTCTGCAGTTACGCCGATTTGTAACGCGATG	Detection of the presence of pBluescriptKS(+) sequence
mpm13e	ATGAATTCATGTCCGCGGACACCGCCAA	
mpm11e	CCGAATTCATGCGTAACGCGTAGGATTGGA	Detection of the entire Φ6 genome sequence
mpm13p	GCGTGCAGTTAATCATGTTTATTTTC	
AK 300	AGTTGGATCCGATAAATTAACCCCGGAG	Detection of the chromosomal gene V*ngo*302
AK 400	GCATCAAGCCTTTCATCTTGTTTGGATGATTTC	
pBSBlaF	TTACCAATGCTTAATCAGTG	Detection of the *bla* gene from pBluescript KS(+)
pBSBlaR	TATGAGTATTCAACATTTCC	
ORF5Nhe	ACTAGCTAGCTACTGCCAAGTCGGAAATAAATG	Detection of *orf5*; cloning of *orf5* in pET28a
ORF5Hind	CCCAAGCTTTCATTTCAACAAAACCTTTTTCAGCAGG	
ORF4NcoI	GGCCATGGATGAAATTTATTAACACCTGCC	Cloning of *orf4* in pET28a
ORF4XhoI	AACTCGAGCTTCATCACCCTCTTAACGA	
ORF7F	CATGCCATGGATGGTCACAAAACATACAAATTTG	Cloning of *orf7* in pET28a
ORF7R	CCCAAGCTTTTCCCCCCCCCAACGAAC	
ORF8NcoI	GGCCATGGATGCCATTACTTTCCGGCCTGA	Cloning of *orf8* in pET28a
ORF8XhoI	GGCTCGAGCCTCATCCCGGAAAGACAAAC	
ORF10NcoI	GCCCATGGATGATTCACAAACCAAGATATATCA	Cloning of *orf10* in pET28a
ORF10XhoI	GCCTCGAGAACAAAATATTTGACTGAAGTCACA	
ORF2NheI	GCTAGCTTTGAAACAAGCCAAGTAACC	Cloning of *orf2* in pET28a
ORF2HindIII	AAGCTTTTAGGCTTTAGGTGCTGCGC	
FKSf1	GGCGAACGTGGCGAGAAAG	Making deletion of *ori* of fd in pBluescript KS(+)
RKSf1	GCCGATTTCGGCCTATTGG	
6LFFOR	GAGGTCGACTATCTGGGCATTCGCTCGGTCAAAC	Cloning of NgoΦ6 with the deletion of *orf7*
6LFREV	GCCTGCAGACTTTCCGGCCTGATTCCACTTTTTAG	
6RFREV	ATGCGGCCGCGTATGGCATACACATCAACAA	
6RFFOR	GCCTGCAGCAATTATCGACAATTTCGCAAAATTCA	
HF7F	TTGTGAGCGGATAACAATTC	Cloning of fusion of *orf7*::HisTag
HF7Rev	AGTTATTGCTCAGCGGTGGC	
Fus5	CGTTCAAGGATTCTTTAATTACCATTTCCG	Construction of pNgoΦ6::BL::*orf7*::HisTag
Fus6	ATGCCATTACTTTCCGGCCTGATTC	
CORF9F	TAATAAAATTTAATGTACGACAACTGAAGA	Amplification of *orf9*
CORF9R	GGGGACTTTCCGCCCATTACGAG	
VECOF	ATAGCTAGCGCCCACGATAAGGCCACTG	Detection of *ecoKDcm*
VECOR	ATAGCTAGCGCCTCAAGCGAGTAAATGAATCC	
DAMF	ATGAAGAAAAAATCGCGGCTTT	Detection of *ecoKDam*
DAMR	TTATTTTTTCGCGGGTGAAACGA	
6LFFOR	GAGGTCGACTATCTGGGCATTCGCTCGGTCAAAC	Cloning of NgoΦ6 fragments for the deletion of *orf7*
6LFREV	GCCTGCAGACTTTCCGGCCTGATTCCACTTTTTAG	
6RFREV	ATGCGGCCGCGTATGGCATACACATCAACAA	
6RFFOR	GCCTGCAGCAATTATCGACAATTTCGCAAAATTCA	

natural host, including *E. coli*, *Haemophilus influenzae*, *Neisseria sicca*, *Pseudomonas* sp., and *Paracoccus methylutens*. We also demonstrate that phage derived from *E. coli*(pBS::Φ6) are able to infect these strains, indicating that NgoΦ6 represents the first characterized broad-host-range filamentous phage.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and growth conditions. *Escherichia coli* Top10, F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80 Δ*lac* Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*araA-leu*) 7697 *galU galK* λ^s*-rpsL endA1 nupG*; *E. coli* strain DH5α, F$^-$ φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ*-thi-1 gyrA96 relA1*; *E. coli* XL1, *recA endA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *supE44 relA1* λ$^-$ Δ*lac* [F′ *proAB lacI*^{q}*Z*Δ*M15 Tn10*(*tet*)]; and *E. coli* ER2566, F′ *proA*$^+$*B*$^+$ *lacI*^{q}Δ(*lacZ*)M15 *zzf*::*mini-Tn10* (Km^r)-Δ(*argF-lacZ*)*U169 glnV44 e14-*(*McrA-*) *rfbD1? recA1 relA1? endA1 spoT1? thi-1* Δ(*mcrC-mrr*)*114*::*IS10* were grown in Luria-Bertani broth (LB) at 37°C or 30°C (21). Antibiotics included in the medium were used at the following final concentrations (μg/ml): ampicillin 25, 50, or 100; and kanamycin, 10. *N. gonorrhoeae* FA1090 and *N. sicca were* grown at 37°C in standard gonococcal medium (designated GCP if broth and GCK if agar) (Difco) plus Kellogg's growth supplements (22) and 0.042% sodium bicarbonate if in broth or in a CO_2 incubator. *H. influenzae* strain Rd was grown at 37°C in brain heart infusion (BHI; Difco) supplemented with 2 μg of NAD/ml and 10 μg/ml of hemin (23). *Pseudomonas* sp. and *P. methylutens* were grown in LB at 37°C or 30°C. Plasmid pBluescript KS(+) was purchased from MBI Fermentas.

DNA manipulations. Genomic DNA was extracted from cultures using the Genomic Mini DNA purification kit (ABA Biotechnologies). Plasmid DNA was purified using the GeneJet Plasmid Miniprep kit (Fermentas), and PCR products were purified using the GeneJet PCR purification kit (Fermentas) according to the manufacturer's instructions. Phage and phagemid DNAs were isolated using the QIprep Spin M13 kit (Qiagen). Testing for the presence of the prophage sequences in genomic or phage DNAs by PCR was performed with primers described in Table 1. PCRs were performed in 50-μl reaction mixtures containing 300 nM the forward and reverse primers, 200 μM (each) deoxynucleoside triphosphates

(Fermentas), 0.5 units of *Taq* or *Pfu* polymerases (Fermentas) in the supplier's buffer, and 100 ng of DNA as the template. Reactions were performed in an MJ Mini thermocycler (Bio-Rad). The specificity of the PCR products was confirmed by DNA sequencing of the amplicons. All routine cloning procedures were carried out in accordance with protocols described by Sambrook and Russell (24). PCR products and other DNA samples were subjected to electrophoresis in 1% agarose gels and stained with ethidium bromide. For DNA/DNA hybridizations, undigested or digested genomic DNAs were subjected to gel electrophoresis and transferred to a positively charged nylon membrane (Millipore). Hybridizations were performed with the DIG High-Prime labeling and detection kit (Roche), using a 220-bp *orf4* PCR amplicon as a probe (obtained with primers ORF4Xho and ORF4Nco).

Construction of recombinant plasmids. To remove the f1 origin of replication from pBluescript II KS(+), localized between nucleotides (nt) 71 and 331, a PCR was performed using primers FKS-f1 and RKS-f1. The amplicon was purified, digested, ligated, and transformed into *E. coli* Top10, selecting for ampicillin-resistant colonies. Plasmid DNA was isolated, and the presence of the correct deletion was verified by restriction enzyme analysis. One plasmid, named pBS KS(+)*, was sequenced to confirm its genetic map. This plasmid was used to clone the NgoΦ6 genome from *N. gonorrhoeae* FA1090.

To clone the *N. gonorrhoeae* DNA fragment carrying the NgoΦ6 DNA sequence (coordinates 1079580 to 1089166) from FA1090 (GenBank accession number AE004969), the PCR product obtained with Phi6F and Phi6R primers was cloned into the BamHI site of pBS KS(+)* plasmid, generating pBS::Φ6. To construct pBS::Φ6Δ*orf11* with the deletion of *orf11*, pBS::Φ6 was cleaved with XhoI and BspBI, the ends were filled with Klenow polymerase, and the fragments were allowed to self-ligate, generating the plasmid pBS::Φ6Δ*orf11* (Fig. 1).

To construct a plasmid containing the NgoΦ6 genome without the *orf7* gene, a fragment of *N. gonorrhoeae* (coordinates 1079656 to 1083640, left arm of the NgoΦ6 genome) was amplified using primers 6LFFOR and 6LFREV and cloned into the PstI and SalI sites of pBS KS(+)*, generating plasmid pBSNgoLFΦ6. The right arm of the phage (coordinates 1085181 to 1088990) was amplified using primers 6RFREV and 6RFFOR and cloned into the PstI and NotI sites of pBSNgoLFΦ6, generating the plasmid pBS::Φ6Δ*orf7* (Fig. 1). DNA sequence analysis confirmed the genetic integrity of the phage genome.

The plasmid pBS::Φ6Δ*orf* 7::*gfp* was constructed by cleavage of the pBS::Φ6Δ*orf7* with PstI and insertion of a *gfp* amplicon (obtained with the use of primers GFPFOR and GFPREV) into this site.

Enzymes and chemicals. Restriction enzymes were purchased from MBI Fermentas and New England BioLabs. T4 DNA ligase, *Pfu* DNA, Tag polymerases, and DNA and protein size markers were purchased from MBI Fermentas. Kits for DNA purification and plasmid DNA isolation were purchased from A&A Biotechnology (Gdansk, Poland). All chemicals used were reagent grade or better and were obtained from Sigma (St. Louis, MO), unless otherwise noted.

Phage and phagemid particle preparation. Overnight cultures were diluted 50-fold into 200 ml of an appropriate medium and grown with shaking at 30°C or 37°C until the optical density at 600 nm (OD_{600}) was 1. In some experiments, mitomycin C (final concentration, 0.3 μg/ml) was added, and incubation continued with shaking overnight. If mitomycin C was not added, cells were grown overnight. Bacteria were collected by centrifugation (20 min at 7,000 rpm), and the supernatant was filtered through a 0.8-μm filter. The filtrate was mixed with 1/5 volume of a solution containing 20% polyethylene glycol (PEG-8000) and 2.5 M NaCl and kept at 4°C overnight to precipitate the phage particles. The precipitate was collected by centrifugation, dissolved in 4 ml of phosphate-buffered saline (PBS), and treated with DNase I and RNase A (25 μg/ml each) for 3 h at 20°C. Phage particles were purified on a QMA-Sephacell column (2 cm by 10 cm) (25).

Infection assays. Infection experiments with *E. coli* cells were performed by mixing, into a final volume of 200 μl of LB medium, 10 μl of phage preparation with 4×10^8 CFU of different recipient bacteria grown at 37°C. After 2 h of incubation, 10-fold serial dilutions were plated onto agar containing the appropriate amount of ampicillin. After 24 h of incubation, the number of Amp^r colonies was determined. The *E. coli* Amp^r colonies were replica plated onto LB, and LB Amp plates and the colonies were grown on LB plates used for further studies. For the plate spot infection, overnight cultures of appropriate bacterial cells (6×10^8 CFU) were used to inoculate 3 ml of semisolid agar medium, poured over appropriate agar medium-containing plates. After the agar overlay solidified, 10 μl of phage suspension was spotted and allowed to dry. Plates were incubated overnight and 30°C or 37°C and then at room temperature for 24 h. A sample of cells from the center of the infected spot was plated onto fresh medium, and after overnight growth, single colonies were checked by PCR analysis for the presence of NgoΦ6 genomic DNA.

Electron microscopy. Overnight cultures of *E. coli*(pBS::Φ6), *N. sicca* (NgoΦ6fm), *H. influenzae* (NgoΦ6fm), *Pseudomonas* (NgoΦ6fm), and *Paracoccus* (NgoΦ6fm) were grown in appropriate media and temperatures, diluted 30 times into fresh media, and incubated with shaking for 2 h. Mitomycin C was added (final concentration, 0.3 μg/ml), and the culture was incubated in the dark for 3 h. Chloroform was added, and the culture was shaken for 20 min. The cells and debris were removed by centrifugation for 20 min at 5,000 rpm, and the supernatant was filtered through a 0.8-μm filter. Phage particles were precipitated by the addition of NaCl to a final concentration of 1 M, polyethylene glycol 8000 to 10%, and $CaCl_2$ to a final concentration of 1 mM. After collection of the precipitate by centrifugation, the precipitate was dispersed in buffer, DNase I and RNase A were added (25 μg/ml), and the solution was incubated for 16 h at 4°C. The solution was centrifuged for 30 min at 12,500 rpm in a Beckman JA20 rotor at 4°C, the pellet was resuspended in Tris-EDTA (TE) buffer, and the solution was applied to a QMA Sephacell column (2 by 15 cm) (25). An aliquot of the eluate was stained with uranyl acetate (2%) for 30 s prior to visualization on a Zeiss EM10CA microscope (80 kV).

RESULTS

Genomic organization of NgoΦ6. The *N. gonorrhoeae* filamentous prophage carried by strain FA1090, designated NgoΦ6 (6) or Nf4-G2(inv) (5), is encoded by an 8.2-kb DNA fragment that is predicted to encode 13 open reading frames (ORFs). The genetic organization of NgoΦ6 resembles the genetic organization of Nmf, a filamentous bacteriophage integrated into several *N. meningitidis* strains (7). The data presented in Fig. 1 illustrate its modular organization, indicating that the putative genes responsible for DNA replication, structure, and assembly are organized in blocks. However, NgoΦ6 lacks an equivalent of the M13 *gpIV* gene (present in Nmf phage as *orf9*) responsible for release of the phage from the cell (7). Computational analysis predicted the integration sites of the filamentous phages present in *N. gonorrhoeae* and *N. meningitidis* (Fig. 1) (5, 7). Kawai et al. (5) postulated that when these phages are integrated into the host chromosome, the integration sequence is divided into two parts located at the end of *orf13* (sequence, 5′-CTTATAT-3′) and the beginning of *orf11* sequence ATCT (Fig. 1). Their data further indicate that in the mature phage particle, the junction would have the sequence ATCT TATAT; this was also predicted to be a part of the main phage promoter sequence (Fig. 1) (5). This suggests that the phage promoter sequence would be disrupted and be inactive when the DNA is present in the prophage state. Kawai et al. also predicted that Nf4-G2(inv) *orf11* encoded a protein responsible for the integration of the phage genome into chromosomal DNA (5).

***E. coli* strains carrying pBS::Φ6 produce phage.** In *N. gonorrhoeae*, DNA can be detected in the released phage particles (6). However, due to the high similarity of the genomes of all four filamentous prophages present in *N. gonorrhoeae* (both at the

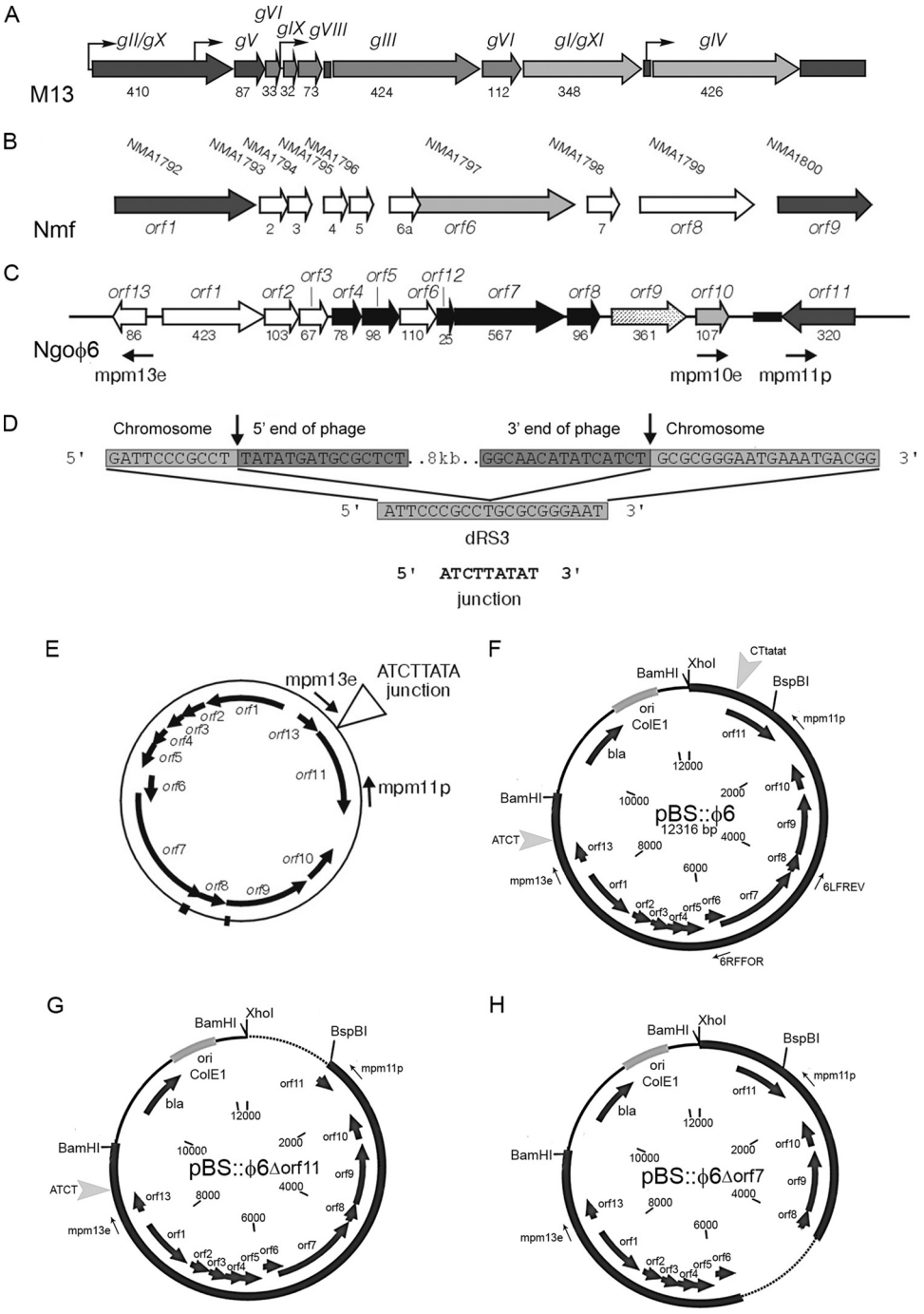

FIG 1 (A, B, and C) Genomic organization of NgoΦ6: M13 filamentous phage of *E. coli* (A); filamentous phage Nf1A present in the chromosome of *N. meningitidis* (7) (B); NgoΦ6 as present on the *N. gonorrhoeae* chromosome (C). The open arrows represent the replication module; the solid arrows represent the structural module; and the arrow with crosshatching represents an assembly module. The arrows with the designations mpm10e, mpm11p, 6LFREV, 6RFOR, mpm13e, mpm11e, and mpm13p show the localization of the specific primers (Table 1) used for detection of pBluescript KSII(+) in the phage genomes. (D) DNA sequence at the phage insertion site of NgoΦ6. The top sequence indicates the sequence of the FA1090 chromosome. The arrows indicate the junction depicting the site of phage insertion into the chromosome. The sequence identified as dRS3 is the predicted sequence that would be found in the chromosome after phage excision. The sequence listed as junction is the DNA sequence found in the bacteriophage genome, as determined from phage particles. (E) Hypothetical circular form of NgoΦ6. The sequence identified by the triangle (ATCTTATA) indicates the genomic organization of the phage integration sequence when contained in the bacteriophage. (F) Map of NgoΦ6fm. The two grey arrows outside the circumference indicate the organization of the DNA sequence, as contained in NgoΦ6fm. Black arrows inside the circumference are oriented in the direction of transcription and represent ORFs. (G) The plasmid pBS::Φ6Δ*orf11* was constructed by cleavage of pBS::Φ6 with XhoI (site located within the polycloning region of pBluescript) and BspB1 located within the *orf11* DNA sequence. The sticky ends were filled out by Klenow polymerase and ligated. (H) pBS::NgoΦ6 was constructed with the deletion of the *orf7* containing the PstI restriction site (forward) and SalI (reverse). The cloned amplicon (3,800 bp long) located between nt 1085181 and 1088990 on *N. gonorrhoeae* chromosomal DNA was inserted into PstI and NotI sites on pBS::Φ6* within the polycloning site. The clone containing the left arm of NgoΦ6 was selected and designated pBS::NgoLFΦ6. The primers for cloning of the right arm of NgoΦ6 contained the site for PsrtI (forward) and NotI (reverse) that allowed to clone a fragment of the *N. gonorrhoeae* genome (nt 1985181 to 1088990) on the PstI and NotI sites of pBS::NgoLFΦ6, generating plasmid pBS::Φ6Δ*orf7*. (G and H) A dotted line indicates the region that was deleted.

DNA and at the protein level) (5, 6), the products of these genes and/or the genes themselves could be present in both phage particles as a result of genetic recombination and/or phenotypic mixing between different phages. To determine if one phage genome was sufficient for phage production, we constructed a clone that contained only the NgoΦ6 genome. The vector pBluescript II KS(+) was modified by deleting the DNA sequence containing the filamentous phage f1 origin of replication, giving pBS KS(+)*. A 9,580-bp PCR product, derived from the chromosome of *N. gonorrhoeae* strain FA1090 (coordinates 1079580 to 1089160) was cloned into the BamHI site of pBS KS(+)* in such an orientation that all the phage genes except *orf11* would be antiparallel to the plasmid *lacZ* promoter, giving rise to plasmid pBS::Φ6 (Fig. 1). This amplicon contains not only all annotated phage *orfs*, together with the junction sequences, but also an additional 640 bp of chromosomal DNA derived from the left and right flanking regions at the ends of the prophage DNA sequence. Hence, this plasmid imitates the chromosomal organization of this phage in the gonococcal genome.

Because pBS::Φ6 contains the gene encoding β-lactamase, we were able to identify transformants containing the entire phage genome on a plasmid by isolating ampicillin-resistant transformants and analyzing them for the presence of the NgoΦ6 genes. One colony, designated *E. coli* Top10(pBS::Φ6), was used for further studies. The integrity of the prophage DNA sequence on this plasmid was confirmed by PCR, restriction digestion analysis, and finally by DNA sequencing (data not presented).

In this construct, the prophage DNA contained on the phagemid mimics the prophage DNA integrated into the chromosomal host DNA; the junction sequences are located on both ends of prophage sequence (Fig. 1), and the predicted promoter is inactivated due to its division into two parts. To determine if phage gene expression can be directed from an internal phage promoter, a derivative of pBS::Φ6 with the DNA sequence containing a promoterless *gfp* gene instead of *orf7* was constructed and transformed into *E. coli* Top10 cells. The transformants expressed *gfp* (data not presented), demonstrating the presence of an additional functional promoter within the phage genome.

To test whether the *E. coli* Top10 strain carrying pBS::Φ6 is able to produce phage particles, the bacterial culture was grown overnight and the presumptive phage particles were purified. PCR analysis using the DNA isolated from such a suspension as the template and two primers, mpm11e and mpm13p, designated to detect only the phage DNA sequence, produced a product corresponding to the length of the whole NgoΦ6 DNA (Fig. 2A). Further analysis showed that this DNA contained the DNA sequence encoding β-lactamase (Fig. 2B, lane 5) as well as the whole sequence of pBS KS(+)*, as judged by the presence of a 4,800-bp PCR amplicon obtained with the primers mpm11p and mpm13e (Fig. 2C). The absence of a PCR product when primers specific for genomic genes *ecoKDcmV* (Fig. 2B, lane 1) and *ecoKDam* (Fig. 2B, lane 2) were used demonstrates that the amplicons were generated from phage-derived DNA and not from contaminating chromosomal DNA. The DNA present in the phage particles was sensitive to S1 nuclease (Fig. 2D, lane 1), indicating the ssDNA character of phage DNA. Since the phage particles obtained from the supernatant of *E. coli*(pBS::Φ6) contain both plasmid and phage DNA sequences, they can be defined as phagemid particles, and we have designated them NgoΦ6fm.

Host requirements of NgoΦ6fm. Since *E. coli* cells carrying pBS::Φ6 produced phagemid particles, we wished to determine if

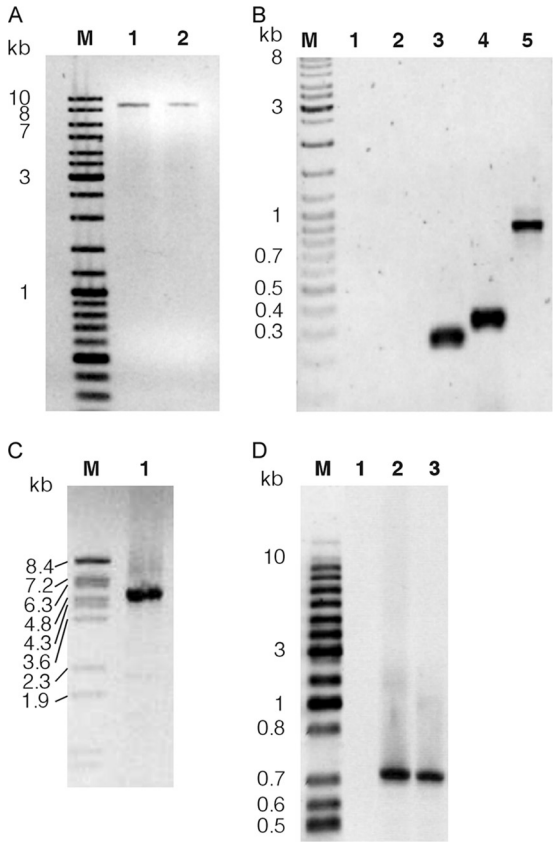

FIG 2 Analysis of phagemid DNA. (A) Amplicons were obtained with primers Phi6F and Phi6R. Lane 1 used genomic DNA as the source for PCR. Lane 2 used DNA isolated from phage particles purified after induction of *E. coli* Top10(pBS::Φ6) as the source of DNA. (B) PCR amplicons obtained with primers specific to *E. coli* gene *ecoKDcmV* (lane 1), *E. coli* gene *ecoKDam* (lane 2), NgoΦ6 *Orf4* (lane 3), *Orf10* (lane 4), and *bla* gene present in pBluescript KSII(+) DNA (lane 5) (see Table 1 for primer sequences). (C) PCR amplicon obtained with primers (mpm11p and mpm13e) specific for the detection of the whole sequence of pBluescript KSII(+) DNA. (D) NgoΦ6fm particles contain (+) ssDNA of NgoΦ6 phage DNA. PCR amplicons obtained with primers COPRF9F and CORF9R: lane 1, DNA treated with S1 nuclease prior to PCR; lane 2, PCR amplicon treated with restriction endonuclease AciI; lane 3, PCR amplicon undigested. Markers are derived from a DNA ladder (1 to 10 kb) (A, B, and D) or phage lambda DNA digested with BstNI (C).

NgoΦ6fm could infect *E. coli*. We infected *E. coli* strains with NgoΦ6fm particles and measured the frequency of infection as the acquisition of the ability to form colonies on medium containing 30 μg/ml of ampicillin, using a standard volume of purified phage. As shown in Table 2, NgoΦ6fm was infectious for *E. coli* Top10, irrespective of F pilus expression. The fact that strains Top10 F^-, DH5αMCR F^-, and XL1 F^- can be infected indicates that NgoΦ6fm does not require the F pilus as a bacterial receptor to infect this strain. This conclusion was further substantiated by the fact that *E. coli* cells carrying pBS::Φ6 with the deletion of *orf7* (Table 2) or a substitution of *gfp* for *orf7* (data not shown) were able to produce phagemid particles that infected *E. coli* cells with the same frequency as the wild-type phage (data not shown). Based on the size of the Orf7 gene and on its position in the genome, and taking into account the similar genetic structures of this family of phages, we hypothesize that Orf7 is probably the adhesin. However, since Orf7 lacks homology with pIII, a protein

TABLE 2 Frequency of infection of *E. coli* strains with NgoΦ6fm and pBS::Φ6Δ*ORF7* NgoΦ6fmΔ*ORF7*

E. coli strain	Mean infectivity rate (± SD)[a]		
	NgoΦ6fm	NgoΦ6fmΔ*orf7*	NgoΦ6fmΔ*orf11*
Top10 F$^-$(pMPMK6Ω)	$(1.4 \pm 0.5) \times 10^{-2}$	$(2.3 \pm 0.5) \times 10^{-2}$	NT[b]
Top10 F$^-$	$(2.1 \pm 0.7) \times 10^{-2}$	$(2.4 \pm 0.7) \times 10^{-2}$	NT
Top10 F′(pMPMK6Ω)	$(1.3 \pm 0.6) \times 10^{-2}$	$(3.2 \pm 0.6) \times 10^{-2}$	NT
DH5αmcr F$^-$	$(2.3 \pm 0.1) \times 10^{-2}$	NT	NT
Top10 F′	$(2.4 \pm 0.3) \times 10^{-3}$	NT	$(1.2 \pm 0.4) \times 10^{-2}$

[a] Infectivity rate is defined as the ratio of the number of ampicillin-resistant colonies to the total number of colonies. Values are from 3 independent experiments.
[b] NT, not tested.

necessary for adsorption of the fd series of phage particles to bacterial F pilus (2), our results indicate that NgoΦ6fm infects cells through a different mechanism.

To verify that the resultant phenotype was not due to a low level of transformation by phage DNA, we treated the phage preparations with DNase. This treatment had no effect on the number of antibiotic-resistant colonies that were obtained (data not shown). Treatment of these preparations with proteinase K eliminated the ability of the preparation to infect *E. coli* Top10 (data not shown).

Because infection by NgoΦ6fm did not need a specific pilus, we hypothesized that NgoΦ6fm might be capable of infecting other Gram-negative bacteria. To test the ability of NgoΦ6fm to infect other Gram-negative bacteria, phagemid particles obtained from *E. coli* Top10(pBS::Φ6) were spotted onto the lawn of bacteria to be infected. After 24 h of incubation, the cells from the center of the infection were spread onto appropriate media to obtain single colonies and then tested for the presence of NgoΦ6 DNA sequences by a colony PCR method. Strains of *E. coli*, *H. influenzae* Rd30, *N. sicca*, *Pseudomonas* sp., and *P. methylutens* carrying NgoΦ6fm DNA sequences were obtained. PCR analysis of the total cellular DNA from *E. coli*, *H. influenzae* Rd30, *N. sicca*, *Pseudomonas* sp., and *P. methylutens* infected by NgoΦ6fm showed the presence of amplicons corresponding to the whole genome of NgoΦ6 DNA (Fig. 3A) and to the β-lactamase gene (Fig. 3B).

Orf7 is not required for infection of *E. coli*, *H. influenzae* Rd30, *N. sicca*, *Pseudomonas* sp., and *P. methylutens*. To demonstrate that Orf7 was not needed to initiate infection, *E. coli*, *H. influenzae* Rd30, *N. sicca*, *Pseudomonas* sp., and *P. methylutens* were infected with NgoΦ6fm::*gfp*, a phagemid construct in which *orf7* was replaced with *gfp*. As was seen in the case of infection of *E. coli* strains, all transfectants contained the *gfp* gene (Fig. 4).

Strains infected with NgoΦ6fm produce functional phagemid particles. To demonstrate the ability of transfected strains to produce phage, bacterial cultures were grown to mid-logarithmic phase and induced (or not induced) with mitomycin C, and potential phage particles were concentrated with PEG and NaCl and purified on Sephacell QMA columns. (We noted that induction of phage with mitomycin was not necessary but that it increased the yield of phage particles [data not shown].) Aliquots of culture supernatants from all bacterial strains carrying the phagemid were analyzed by electron microscopy. The data in Fig. 5 show that phage particles could be identified in supernatants isolated from cultures of *E. coli* Top10(pBS::Φ6fm) (Fig. 5A), *N. sicca* (NgoΦ6fm) (Fig. 5B), *H. influenzae* Rd30 (NgoΦ6fm) (Fig. 5C), *Pseudomonas* sp. (NgoΦ6fm) (Fig. 5D), and *P. methylutens* (NgoΦ6fm) (Fig. 5E). All preparations showed the presence of morphologically similar (about 4-μm-long) filamentous phage particles. All of them also showed the presence of the whole phagemid DNA together with the pBluescript DNA (data not shown). NgoΦ6fm particles isolated from each of the bacteria tested were able to transfect other bacteria, producing the next generation of active phagemid particles (data not shown).

Location of pBLNgoΦ6 phagemid genome in different Gram-negative bacteria. The ssDNA of M13 phage, upon infection of *E. coli*, is immediately transformed into a replicative form of double-stranded DNA (dsDNA), establishing a stable pseudolysogeny state inside the cell (26). The phage genes are expressed, and progeny phage ssDNA is packaged into phage particles. This is contrasted with phage CTXΦ, whereby its genomic ssDNA is introduced into the cell as an ssDNA, transformed into

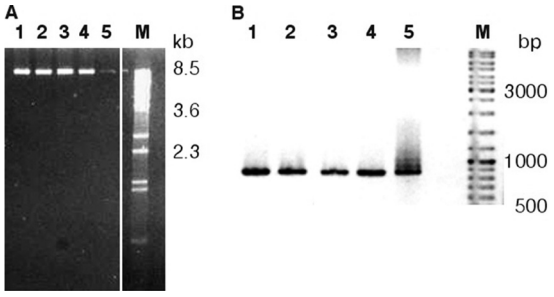

FIG 3 Presence of phagemid DNA in infected bacterial cells. Various bacterial strains were infected with NgoΦ6fm. Lanes 1 to 5 used the total DNA isolated from *E. coli* Top10, *H. influenzae* Rd30, *N. sicca*, *Pseudomonas* sp., and *Paracoccus methylutens* cells as the source for PCR. (A) Amplicons were obtained with primers Phi6F and Phi6R. (B) PCR amplicons obtained with primers specific to the β-lactamase gene.

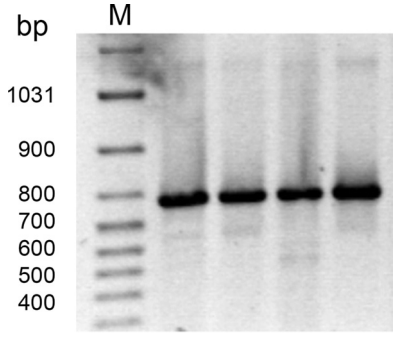

FIG 4 Requirement of Orf7 for initiation of phage infection. Various bacterial strains were infected with phage NgoΦ6fm::GFPΔ*orf7*. Lanes 1 to 5 used the total DNA isolated from phage-infected *E. coli* Top10, *H. influenzae* Rd30, *N. sicca*, *Pseudomonas* sp., and *Paracoccus methylutens* cells as the source for PCR. PCR amplicons with primers specific for *gfp* gene are shown. Numbers on the left represent molecular markers in bp.

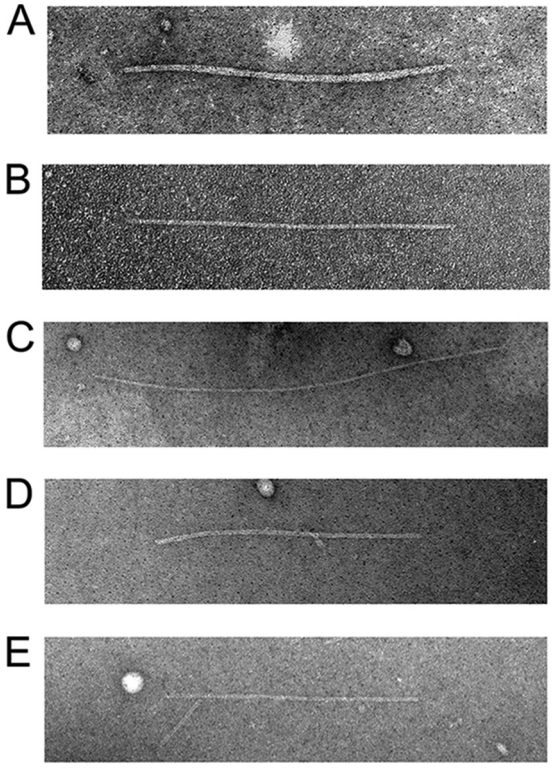

FIG 5 Transmission electron micrograph of phagemid particles isolated from various bacterial species. Culture supernatants were precipitated with PEG 8000 and NaCl, purified on Sephacell QMA columns, dialyzed against TE buffer, added to a gold grid, stained with uranyl acetate, and visualized on a Zeiss EM10CA electron microscope. Particles were obtained after induction of *E. coli* Top10 (NgoΦ6fm) (A), (B) *N. sicca* (NgoΦ6fm) (B), *H. influenzae* Rd30 (NgoΦ6fm) (C), *Pseudomonas* sp. (NgoΦ6fm) (D), and *Paracoccus methylutens* (NgoΦ6fm) (E). Bar, 1.2 μm.

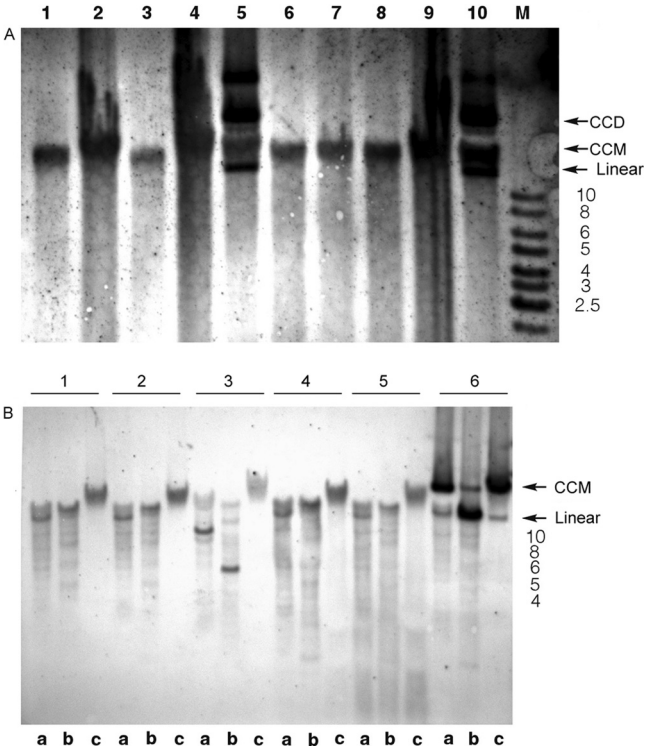

FIG 6 Cellular location of the acquired phage DNA. (A) Southern hybridization using *orf4* as a probe against undigested total DNA isolated from various strains carrying NgoΦ6 phage derivatives. Lanes: 1, total DNA from *E. coli* DH5α infected with NgoΦ6fm; 2, total DNA from *E. coli* XL1 infected with NgoΦ6fm; 3, total DNA from *E. coli* Top10 infected with NgoΦ6fm; 4, total DNA from *H. influenzae* Rd30 infected with NgoΦ6fm; 5, total DNA from *E. coli* XL1 transformed with pBS::Φ6 plasmid; 6, total DNA from *N. sicca* infected with NgoΦ6fm; 7, total DNA from *Pseudomonas* infected with NgoΦ6fm; 8, total DNA from *Paracoccus* infected with NgoΦ6fm; 9, total DNA from *E. coli* DH5α infected with NgoΦ6fm; 10, total DNA from *E. coli* DH5αMCR transformed with pBS::Φ6 plasmid. M, molecular weight markers (in thousands). (B) Southern hybridization using *orf4* as a probe against digested genomic DNA isolated from various strains carrying Ngo6Φfm. a, digested with BglII; b, digested with ScaI; c, nondigested. Set 1, total DNA from *E. coli* XL1 (NgoΦ6fm); set 2, total DNA from *H. influenzae* (NgoΦ6fm); set 3, total DNA from *N. sicca* (NgoΦ6fm); set 4, total DNA from *Pseudomonas* (NgoΦ6fm); set 5, total DNA from *Paracoccus* (NgoΦ6fm); set 6, plasmid NgoΦ6fm DNA isolated from *E. coli* Top10 transformed with pBS::Φ6 plasmid DNA.

dsDNA, and immediately integrated into the chromosome (27). For other filamentous phages, the DNA can exist inside the bacterial cells in several guises: free phage DNA without integration, coexistence of replicating dsDNA phage form and as an integrated copy of phage genome, or as an integrated form of the genome without phage production and no plasmid form of phage genome (8, 11).

To determine the fate of the phagemid DNA acquired either by transformation of the dsDNA in *E. coli* or by infection by the phagemid particles in a broad spectrum of Gram-negative bacteria, total DNA was isolated, separated on an agarose gel, and subjected to Southern hybridization with *orf4* as a probe. This probe never hybridized with the DNA of any of the recipients prior to infection, indicating an absence of background signal (data not shown). Additionally, DNA sequence analysis of available genomic sequences of *E. coli* and *H. influenzae* strain Rd indicates that these strains do not contain any DNA sequences homologous to *orf4* (data not shown). The data indicate that in *E. coli* cells that acquired pBS::Φ6 by transformation, the *orf4* probe hybridizes with two forms of DNA: plasmid DNA and chromosomal DNA (Fig. 6A, lanes 5 and 10). When transfectants were obtained using NgoΦ6fm particles, a signal specific for *orf4* was seen in all strains but was detected only as hybridizing with the chromosomal DNA. No hybridizing signals corresponding to plasmid DNA or any lower-molecular-weight chromosomal DNAs were observed (Fig. 6A, lanes 1 to 4 and 6 to 9). The plasmid form of this phagemid could not be introduced into the various strains via transformation, most likely due to the fact that the Bluescript base is incapable of replicating in *H. influenzae* Rd30, *N. sicca*, *Pseudomonas* sp., and *P. methylutens* (D. C. Stein, unpublished observations).

To confirm that the DNA in the DH5αMCR transformed with pBS::Φ6 corresponded to dsDNA plasmid DNA, DNA was isolated from a transformant and digested with ScaI (recognizing one site in the phage part of the phagemid molecule) or BglII (an enzyme that has no recognition site in the phagemid molecule). The data (Fig. 6B, set 6a) indicate that when DNA was digested with BglII, an enzyme whose recognition sequence is not present in the vector base nor in the NgoΦ6 part of pBS::Φ6, the one predominant fragment visible corresponded to the mobility of the undigested supercoiled monomer. When this DNA was digested

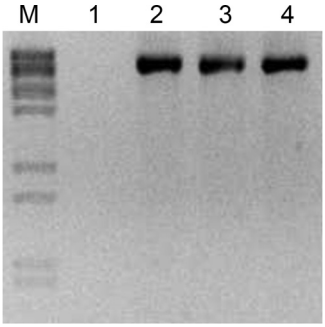

FIG 7 Detection of phagemid pBS::Φ6fm DNA in the chromosome of cells infected with phagemid mutant lacking the *orf11* integrase gene. Lane 1 used DNA isolated from of *H. influenzae* Rd30 (NgoΦ6fm::Δ*orf11*) as the source for PCR and primers mpm13e and mpm11p. Such amplicons can be formed only from the circular form of phagemid DNA. Lane 2 used DNA isolated from of *H. influenzae* Rd30 (NgoΦ6fm::Δ*orf11*) as the source for PCR and primers mpm11e and mpm13p; such amplicons can be formed with both linear chromosomal and circular forms of DNA. Lane 3 used DNA isolated from *Pseudomonas* sp. (NgoΦ6fm::Δ*orf11*) as the source of DNA, and lane 4 used DNA isolated from *E. coli* (NgoΦ6fm::Δ*orf11*) as the source for PCR in the presence of primers mpm11e and mpm13p. Markers are derived from phage lambda DNA digested with BstNI.

with ScaI, a band of about 12 kb, corresponding to the linear size of the phagemid, was visible (Fig. 6B, set 6b).

If phagemid DNA is integrating into the chromosome in a specific location after transfection, after digestion with ScaI or BglII, one would expect a single predominant band after hybridization. Analysis of DNA isolated from *N. sicca* transfectants appears to produce a single predominant band after digestion with these enzymes. However, multiple faint hybridizing bands are also visible. While this could indicate a low percentage of integration of phage DNA into the chromosome at random sites, we cannot discount that these bands are artifacts of the hybridization. Analysis of the digestion profiles of DNA isolated from the other strains appears to be more heterogeneous, consistent with the random integration of phagemid DNA into various chromosomal locations.

To determine if the integration of NgoΦ6fm into the chromosome of Gram-negative bacteria depends on the integrase encoded by the phage, we constructed a derivative of pBS::Φ6 with deletion of *orf11*, the gene encoding the phage integrase (15, 23). *E. coli* Top10 cells carrying pBS::Φ6Δ*orf11* were grown overnight, and presumptive phage particles were purified and used to infect *E. coli* Top10 F′ on LB plates. The frequency of infection by pBS::Φ6Δ*orf11* was similar to that obtained with NgoΦ6fm (Table 2), demonstrating that this gene is not needed for phagemid replication as a plasmid. To demonstrate that this phagemid could integrate into the chromosome, PCR analysis was performed using two pair of primers. The first one (mpm13p and mpm11e) would generate a product if the phage DNA were integrated into the chromosome or in the circular form. The second one (mpm11p and mpm13e) would generate a product only from the circular form of phagemid DNA. The data in Fig. 7 show the presence of an amplicon of the appropriate size, obtained when the first pair of primers was used, thus verifying the chromosomal location of NgoΦ6fm in all infected species.

DISCUSSION

Bacteriophages contribute to bacterial evolution by mediating horizontal gene transfer and inducing genomic rearrangements. The best-characterized example of a bacteriophage contributing to disease is the emergence of toxigenic strains of *Vibrio cholerae* that contain CTXΦ, a filamentous bacteriophage carrying the genes encoding cholera toxin (28). However, this phage is host restricted and infects only *V. cholerae*. Construction of recombinant phage that had *V. cholerae* phage CTXΦ pIII gene changed the specificity of infection (12). This is due to the fact that the ability of a bacteriophage to infect a bacterial cell and produce progeny phage depends on its ability to adsorb to the cell through specific interaction between phage and cell receptors. The ability of filamentous phage belonging to *Inoviridae* to infect the host depends on specific interactions between phage receptor pIII (a protein with very high homology in all phages of this family). CTXΦ-mediated infections require the binding of the phage to the pilus, with subsequent translocation into the cytosol mediated by the Tol protein (29).

We have shown that NgoΦ6fm, based on the filamentous phage NgoΦ6, can infect and effectively produce progeny phagemids in a broad range of Gram-negative bacteria belonging to the *Alphaproteobacteria* (*Paracoccus*), *Betaproteobacteria* (*Neisseria*), and *Gammaproteobacteria* (*Escherichia*, *Haemophilus*, and *Pseudomonas*). This infection is not mediated through the phage receptor encoded by *orf7* since phagemids deficient of this gene can also infect different bacteria. This indicates that NgoΦ6fm is able to bind to the cell via a mechanism that is independent of the presence of the phage receptor encoded by *orf7*.

The general view of the filamentous phages life cycle was that ssDNA forms have to be converted into dsDNA prior to their integration (30) via different mechanisms including integration into a *dif*-like site by host-encoded XerC/D recombinase (9) or integrating into an RNA gene by the phage-encoded recombinase (31) or the host PivNM/Irg recombinase as postulated for the integration of the *Neisseria* filamentous phages (5). Similar recombinases are used by VEJΦ of *Vibrio parahaemolyticus* (32), Cf1e, Cf1t, Cf1Gv1, and Φ of *Xanthomonas campestris* (33), or YpfΦ of *Yersinia pestis* (8). However, integration of the CTXΦ phage, although carried out by the XerC/D host recombinases, uses ssDNA as a substrate for the integration. Our results indicate that the bacteria are supplying the molecular machinery necessary for production of the progeny phages.

Our data demonstrate that *E. coli* cells carrying pBS::Φ6 produce phage particles and that these phage particles are able to infect different Gram-negative bacteria. The resultant phage all appeared to be morphologically similar, as viewed by electron microscopy. While NgoΦ6 shares the overall genomic organization of phages of the *Inoviridae* family, NgoΦ6 lacks an equivalent of the M13 phage gene IV (gpIV), responsible for secretion of phage particles, suggesting that it uses another bacterial system for secretion. This can explain mechanistically how this phage can be released from different Gram-negative bacteria. These phage sustain biological activity, as evidenced by the fact that they are able to infect different Gram-negative bacteria and produce the next generation of phagemid particles.

Our results also show that the pBS::Φ6 mutant that lacks an active *orf11* encoding PivNM/Irg recombinase homolog is able to form a lysogen and excise from the chromosome, indicating that NgoΦ6 uses a different mechanism for integration and excision. The phagemid NgoΦ6fm cannot establish a stable plasmid molecule after infection but is able to integrate into the host chromosome to form a lysogen. We assume that integration of this mol-

ecule, like the CTXΦ phage, is carried out via its single-stranded genomic form using an unknown integrase. The ability to form infective phagemid particles suggests a potential in transmission of foreign genes and in the evolution of bacteria.

The gonococcus contains numerous restriction/modification systems (34), with NgoI appearing to be closely related to *Haemophilus influenzae* (35) and NgoMIV appearing to be related to enzymes found in a variety of Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Paracoccus* species, etc. (D. C. Stein, unpublished observations). Acquisition of single-stranded DNA through phage infection would bypass most host-mediated restriction systems (36), allowing for acquisition of new traits. The ability of NgoΦ6 to infect a variety of bacterial species suggests a broader role for filamentous phages in bacterial evolution.

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